Ecological and genetic differentiation of two subspecies of Saussurea alpina in the Western Alps

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ORIGINAL PAPER

Ecological and genetic differentiation of two subspecies of *Saussurea alpina* in the Western Alps

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Abstract In the Western Alps, two subspecies of *Saussurea alpina* are found in a partly overlapping distribution area; however, they prefer distinct habitats. While *Saussurea alpina* ssp. *alpina* is distributed throughout the Alps and beyond, *S. alpina* ssp. *depressa* is endemic to the region ranging from the Maritime Alps (France) to the Valais (Switzerland). The sympatric occurrence of closely related taxa raises general questions which factors drive speciation. In order to investigate the genetic and ecological differentiation of the two subspecies, we applied Amplified Fragment Length Polymorphisms and analyzed the habitat preferences via soil pH measurements and vegetation relevés. Overall, we studied 12 populations (five populations of *S. alpina* ssp. *alpina* and seven of *S. alpina* ssp. *depressa*). The populations were found to be genetically highly differentiated ($F_{ST} = 0.42$). Nevertheless, a weak, but significant genetic differentiation between the subspecies could be detected ($F_{CT} = 0.04$) and the results of the ecological analyses showed a clear differentiation in habitat types. While *S. alpina* ssp. *alpina* occurs in alpine grasslands, *S. alpina* ssp. *depressa* occurs mainly on calcareous scree with a significantly higher soil pH (median pH = 7 and median of pH = 8.14 for *S. alpina* ssp. *alpina* and *S. alpina* ssp. *depressa*, respectively) and different surrounding vegetation. We conclude from the clear difference in ecology that ecological plant speciation is a major factor in establishing and maintaining a reproductive barrier. We explain the weak genetic differentiation with a recent separation of the two evolutionary units during the last glaciation cycles or even in postglacial periods.

Keywords Ecological speciation · AFLP · Saussurea · Phylogeography · Incipient speciation · Alps · Soil pH

Introduction

Current distribution patterns of alpine plants are the result of species adaptation to harsh environmental conditions on the one hand (Körner 2003) and on the other hand historical events such as climatic changes and glaciations during the Pleistocene (Comes and Kadereit 1998; Hewitt 2000; Schönswetter et al. 2005). The interaction of these two main drivers of differentiation—ecology and history—often leads to patchy and disjunct geographical distribution patterns (Cox and Moore 1993) and can directly influence intraspecific allele frequencies and hence plant speciation (Thorpe et al. 1994).

Ecological plant speciation is driven by divergent selection due to adaptation to certain ecological factors followed by reproductive isolation (Rundle and Nosil 2005; Givnish 2010). This predicts that a new species might evolve by differentiation of populations adapted to divergent habitats. Alpine ecosystems are characterized by high habitat

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diversity within short distances. It has been shown that the soil pH is a major influence in shaping patterns of species diversity, composition, and distribution at a small as well as large scale (Pärtel 2002; Kerner 1863). Adaptation to different soil types can act first as a key factor for ecological plant speciation followed by maintenance of a reproductive barrier between closely related species because of regional isolation of habitats (Choler et al. 2004). Thus, affinities to different soil types can determine distribution patterns of species and genetic lineages (Kerner 1863; Wolgemuth 2002; Ewald 2003; Alvarez et al. 2009).

A major historical factor shaping distribution patterns of species has been the Quaternary Ice Ages, during which species distributions have undergone drastic range contractions and extensions due to the expansion and subsequent reduction of ice shields in the Arctic and high mountain ranges of the Alps (e.g., Dynesius and Jansson 2000). It has been established that in the Alps alpine species were able to survive in refugia adjacent to ice shields (for a review, see Schönswetter et al. 2005). The dynamics of that process led to habitat fragmentation and range shifts that led to genetic admixture, genetic drift or even allopatric speciation. These processes leave genetic signatures in present day biodiversity. This has been shown in phylogeographical patterns related to refugia and also as modified genetic diversity or rarity, loss of alleles or increased homozygosity (Hewitt 2000; Schönswetter et al. 2005).

The high-alpine and arctic *Saussurea alpina* (L.) DC. (Asteraceae, Carduoideae) is an outbreeding species with a widespread but disjunct distribution ranging from the Pyrenees, the Alps, and the Carpathians to Scandinavia and to the Central Asian Mountains and Northern Siberia (Meusel and Jäger 1992). *Saussurea alpina* is a polymorphic taxon, which shows remarkable variation mainly in shape, size, pubescence, and form of leaf margin, as well as in the leaf shape and pubescence of the phyllaries (Tutin et al. 1976). The morphological variation of *S. alpina* in the Western Alps has led to the recognition of two subspecies: *S. alpina* ssp. *alpina* is usually between 5 and 60 cm tall and the lower cauline leaves are narrowly lanceolate, whereas *S. alpina* ssp. *depressa* is 2–10 cm tall and the lower cauline leaves are broadly lanceolate (Binz and Heitz 1990). *Saussurea alpina* ssp. *alpina* is widespread and ranges from the Eastern Alps to the Upper Savoy, whereas *S. alpina* ssp. *depressa* (Gren.) Nyman is endemic to the Western Alps (Alpes-Maritimes–Valais; Guinochet and de Vilmorin 1982). Although both subspecies are partly sympatric in the Western Alps, they are confined to different habitat types: *Saussurea alpina* ssp. *alpina* inhabits alpine, wind-exposed grassland environments with neutral to slightly acidic soil conditions. The phytosociological associations, in which *S. alpina* ssp. *alpina* is reported, are Elynion and Caricion curvulae (Hegi 1929; Delarze and Gonseth 2008). *Saussurea alpina* ssp. *depressa* is found in the alpine life zone on screes in generally calciphilous vegetation types of the Drabion hoppeanae (Delarze and Gonseth 2008). *Saussurea depressa* Gren. was originally described by J. Ch. M. Grenier (1849) as a Western Alpine species distinct from *S. alpina*, but was later classified as subspecies by C. F. Nyman (1879). We refer to this taxon as subspecies, which is currently accepted in most but not all flora treatments (Binz and Heitz 1990; Aeschimann et al. 2004; Lauber and Wagner 2007; but see Guinochet and de Vilmorin 1982; Hess et al. 2010).

In this study, we investigate if divergent morphology and habitat preferences as well as the restricted distribution pattern of *S. alpina* ssp. *depressa* are the result of a distinct evolutionary history and (incipient) ecological plant speciation. First, we evaluate to what extend the ecology of the two subspecies differ. Second, we test if neutral genetic markers distinguish the two subspecies genetically and third, we evaluate if ecological and genetic differentiation patterns correspond. We approach this research question by investigating ecological habitat preferences of the two subspecies via vegetation relevés and soil pH measurements and by applying AFLPs. Furthermore, we discuss the phylogeographic history of the taxa based on findings from the genetic analyses.

Material and methods

Populations studied

Fifteen populations between the Rhône-Alpes (France) and Grisons (Switzerland) were studied (Table 1; Fig. 1): five populations were identified based on morphology as *Saussurea alpina* ssp. *alpina*, seven as *S. alpina* ssp. *depressa*, and three populations from Eastern Switzerland of the closely related *S. discolor* were included to be able to exclude that gene flow from this species might influence the interpretation of the genetic analyses (Wang et al. 2009; Von Raab-Straube 2003). Voucher specimens are deposited in the Herbarium of the University of Zürich (Z), Switzerland.

Ecological habitat analyses

Vegetation analyses

For every population a vegetation relevé was conducted on 40 × 40 cm plots, each with a *Saussurea* individual situated in the center. The number of plots per population varied between one and three, reflecting the heterogeneity of the vegetation and habitat conditions as thorough as possible. Because *S. alpina* propagates also clonally via subterranean runners, the number of individuals was estimated as the number of rosettes in each population. The species lists

Table 1 List of populations with coordinates, elevation, population size (number of rosettes), area (m^2) and mean soil pH

Population identity	Geographical group	Taxon	Locality/Country	Lat/Long	Elev (m)	Population Size	Area (m^2)	Soil pH	Veg
Sa_CH_Zermatt	C	Alpina	Zermatt/CH	45.99/7.69	2,419	250	2,000	7.31	X
Sa_CH_Moiry	C	Alpina	Lac de Moiry/CH	46.10/7.58	2,410	50	12	7.00	X
Sa_CH_Chanrion	C	Alpina	Chanrion/CH	45.94/7.38	2,488	100	400	6.00	X
Sa_F_Seigne	W	Alpina	Col de Seigne/F	45.74/6.81	2,567	250	75	7.02	X
Sa_F_Aussois	W	Alpina	Aussois/F	45.26/6.71	2,307	200	1,250	5.47	X
Sde_CH_BellaLui	C	Depressa	Bella Lui/CH	46.34/7.49	2,516	800	1,600	8.61	X
Sde_CH_Audannes	C	Depressa	Lac des Audannes/CH	46.35/7.39	2,643	1,000	10,000	8.24	X
Sde_CH_Sanetsch1	C	Depressa	Col de Sanetsch 1/CH	46.33/7.33	2,664	750	1,500	7.93	X
Sde_CH_Sanetsch2	C	Depressa	Col de Sanetsch 2/CH	46.33/7.31	2,579	100	600	8.04	X
Sde_F_Galibier	W	Depressa	Col du Galibier/F	45.05/6.39	2,774	5,000	10,000	8.36	X
Sde_F_Grave	W	Depressa	La Grave/F	45.09/6.32	2,813	1,500	5,000	8.65	X
Sde_F_VillardReymond	W	Depressa	Villard Reymond/F	45.00/6.02	2,387	150	NA	NA	
Sdis_CH_Lukmanier	E	Discolor	Lukmanierpass/CH	46.55/8.81	2,139	60	100	6.41	X
Sdis_CH_Nufenen	E	Discolor	Nufenen/CH	46.54/9.22	1,967	80	100	5.31	X
Sdis_CH_Schwägalp	E	Discolor	Schwägalp/CH	47.24/9.32	1,706	200	600	5.39	X

X in the Veg column indicates if vegetation data of the population are available. Geographical groups among *S. alpine* populations were assigned according to the Aosta break zone (Central vs. Western), *S. discolor* populations are assigned as Eastern (E) group. X in the column 'Veg' indicates if the populations have been included in the ecological analyses

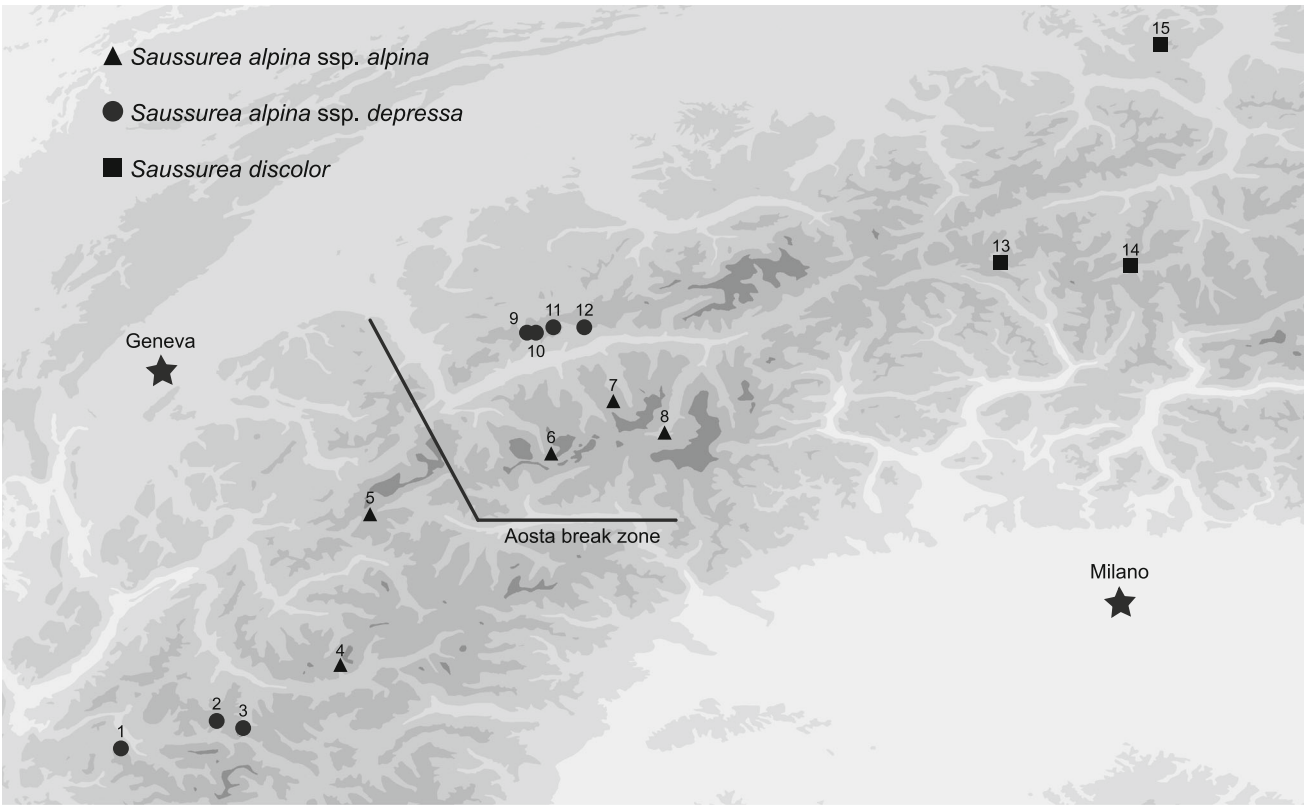

Fig. 1 Sampling of *Saussurea discolor* and *Saussurea alpina* populations in the Western European Alps. 1: Sde_F_VillardRey, 2: Sde_F_Grave, 3: Sde_F_Galibier, 4: Sa_F_Aussois, 5: Sa_F_Seigne, 6: Sa_CH_Chanrion, 7: Sa_CH_Moiry, 8: Sa_CH_Zermatt, 9: Sde_CH_Sanetsch1, 10: Sde_CH_Sanetsch2, 11: Sde_CH_Audannes, 12: Sde_CH_BellaLui, 13: Sdis_CH_Lukmanier, 14: Sdis_CH_Nufenen, 15: Sdis_CH_Schwägalp. The map was generated using DIVA-GIS, version 7.5 (http://www.diva-gis.org)

obtained from the vegetation relevés per population were combined in a presence/absence matrix and a hierarchical clustering method based on average linkage using Jaccard's dissimilarity index was applied with the R package "vegan" (Oksanen et al. 2011). Furthermore, the mean of the indicator values according to Landolt (2010) was calculated for each population and a Principal Component Analysis (PCA) was performed on the data using R development core team (2008).

Soil pH analysis

Soil samples were collected from all sampled plots and were subsequently dried and stored at −80 °C. The pH value was measured about 30 min after adding deionized water to the sample in a 1:1 ratio of weight. The measurements were repeated five times after steering the sample for several minutes. The mean pH value for each population was calculated from the means of the plots and measurements. A Wilcoxon Rank Test and a boxplot were calculated in R development core team (2008).

Genetic analyses

DNA extraction

Total genomic DNA was extracted from silica dried leaf samples from 175 samples using the CTAB protocol (Doyle and Doyle 1987). The quality of the extracted DNA was quality checked on 1 % TAE-agarose gels.

Production of AFLP data

We followed the protocol according to the procedure of Vos et al. (1995) with minor modifications described in the paper of Bendiksby et al. (2011) except that Go-Taq® DNA polymerase (5 U/µl; Promega) was used for PCR amplification. We applied the following selective primer combinations with fluorescent-labeled EcoRI primes: 6-FAM-EcoRI-ACA/MseI-CAC, VIC-EcoRI-ACG/MseI-CTC and NED-EcoRI-ACC/MseI-CAG. The DNA quantity used in the AFLP study was chosen according to the strength of the signal on the agarose gels after extraction, i.e. 10 µl of extracted DNA with thick bands was diluted with 20 µl ddH_20, and extractions showing weak bands indicating low genomic DNA concentrations were used without dilution. Two samples were replicated in each run for estimating an error rate of the AFLPs. The PCR fragments were separated on a 48 capillary sequencer (MegaBACE 1000; GE Healthcare, Chalfont St. Giles, United Kingdom) according to the manufacturer's instructions.

The electropherograms were scored in DAx 8.0 software (Data Acquisition and Data Analysis Software; Van Mierlo Software Consultancy, Eindhoven, The Netherlands) using the MegaBACE ET550-ROX Size Standards (GE Healthcare, Chalfont St. Giles, United Kingdom) as internal size standard. The peak-search function was applied and the peaks were controlled manually in order to be able to even out different peak intensities that may be the result of various technical issues, such as capillary quality or small differences in the PCR reactions (Bendiksby et al. 2011). The scoring bins were set manually, and the resulting presence/absence matrix was exported from DAx 8.0.

The samples were divided into two datasets. Dataset 1 (DS1) refers to the entire dataset including *S. alpina* ssp. *alpina*, *S. alpina* ssp. *depressa* and *S. discolor*. Dataset 2 (DS2) consists of samples of *S. alpina* ssp. *alpina* and *S. alpina* ssp. *depressa* only.

Analyses of AFLP data

Marker frequencies, number of bands per individual, gene diversity index according to Nei (1973, henceforth referred to as genetic diversity) and "frequency-down-weighted marker values" (DW) according to Schönswetter and Tribsch (2005) were calculated in R development core team (2008) using the R-script AFLPdat (Ehrich 2006). As a measure of genetic divergence, the number of unique fragments (F_{uni}) was calculated for each population, and as a measure of within-population diversity, the total number of fragments (F_{tot}) and the number of polymorphic fragments (F_{poly}) per population were calculated. To even out different population sample sizes, only five randomly selected individuals per population were included for the calculations.

The Bayesian clustering method in Structure 2.3.3 (Pritchard et al. 2000) was applied for the DS2 to conduct the population mixture analysis using Structure 2.3.3 with the approach developed for dominant AFLP markers (Falush et al. 2007). The dataset was transformed into an appropriate format using the R-script AFLPdat (Ehrich 2006). The recessive allele model with the admixture model with correlated allele frequencies was applied. We calculated 1×10^6 Monte Carlo Markov Chain iterations and set the burn-into 200,000. A total of 10 replicates from $K = 1$ to $K = 19$ number of groups were calculated on Bioportal (University of Oslo, Norway, http://www.bioportal.uio.no). The $\log_e P(D)$ value of each calculation was extracted in order to evaluate probable solutions of the value K. Moreover, we calculated delta$K$ according to Evanno et al. 2005 with STRUCTURE HARVESTER (Earl and vonHoldt 2012).

An analysis of molecular variance (AMOVA) and Mantel test on DS2 were calculated with the software Arlequin 3.5.1.3 (Excoffier et al. 2005) to quantify genetic differentiation and to test for isolation by distance (IBD). In order to analyze the geographic component in the hierarchical AMOVA, the data were partitioned geographically according to

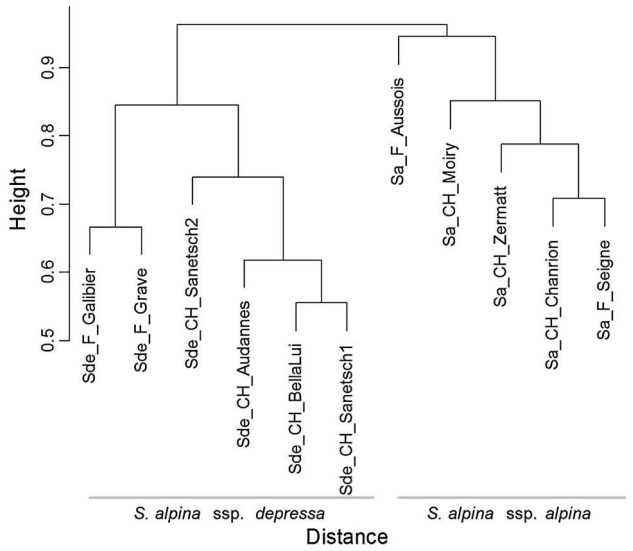

Fig. 2 Hierarchical clustering of populations based on the average linkage clustering method using Jaccard's dissimilarity index (Jaccard 1901)

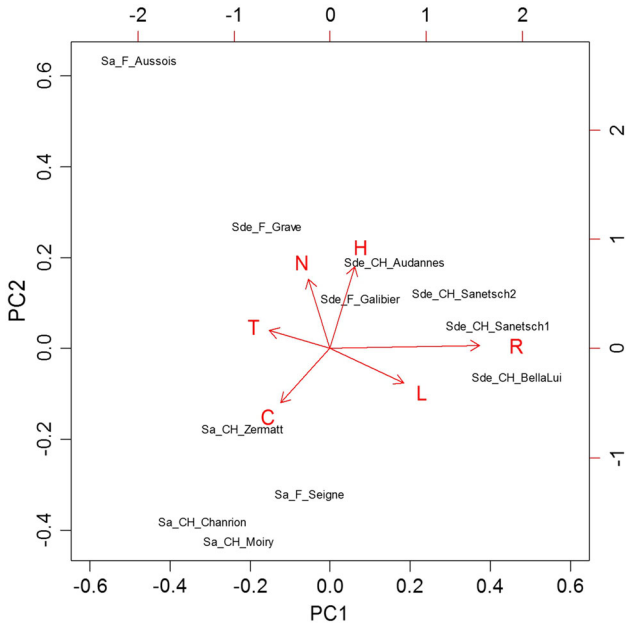

Fig. 3 Principal Component Analysis (PCA) of the mean indicator values (Landolt 2010) per population. *H* humidity, *R* soil reaction, *N* nutrients, *L* light intensity, *T* temperature, *C* continentality, PC1 and PC2 explain cumulatively 88.19 % of total variation

the Aosta break zone of distribution of alleles and species published by Thiel-Egenter et al. (2011) (Fig. 1). Permutation tests as implemented in the Arlequin software (1,023 permutations) for two- and three-level AMOVA were used to examine whether levels of differentiation were significantly greater than zero. The data matrix was transformed to Arlequin data format using the R-script AFLPdat (Ehrich 2006).

A Principal Coordinate Analysis (PCoA) was performed with the inter-individual similarity index of Jaccard (1901) using R. We used the R package "prabclus" (Hennig and Hausdorf 2010) for the calculation of the similarity matrix and the R package "APE" (Paradis et al. 2004) for the PCoA computation and visualization.

Results

Ecological analysis

Vegetation analyses

The hierarchical clustering method based on average linkage using the vegetation relevé data showed that the sites for the *S. alpina* populations clustered clearly in two groups corresponding to the two subspecies (Fig. 2). The PCA with the means of the indicator values according to Landolt (2010) for each population is shown in Fig. 3. The habitat of *S. alpina* ssp. *depressa* tends towards more alkaline, nutrient-rich and humid conditions, whereas *S. alpina* ssp. *alpina* favors a slightly higher continentality.

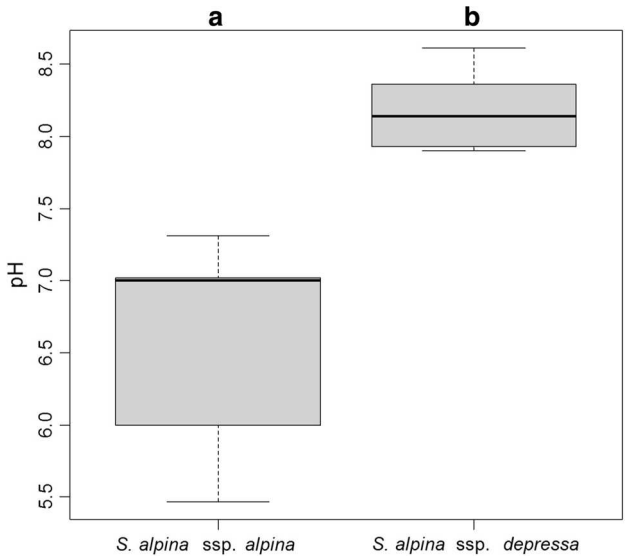

Fig. 4 Boxplot of the mean pH values of the populations ($n = 5$ and $n = 6$ for *S. alpina ssp. alpina* and *S. alpina* ssp. *depressa*, respectively). The *box* represents 50 % of the data and the whiskers are maximum 1.5 times the interquartile range. The *thick line* represents the median

Soil pH values

Our measurements of the soil pH values in 11 populations showed that the substrate of the two *S. alpina* subspecies differ significantly with a median of pH = 7 for *S. alpina*

Table 2 Population identity, number of individuals per population (*N*) and diversity measurements of all populations that were investigated in the AFLP analysis

Population identity	*N*	F_{tot}	F_{Poly} (%)	F_{Uni}	GenDiv	DW
Sa_F_Seigne	9	97	16.6	0	0.08	14.1
Sa_F_Aussois	8	109	37.1	1	0.18	10.8
Sa_CH_Charion	9	126	33.1	0	0.17	21.3
Sa_CH_Moiry	5	111	25.1	1	0.11	23.4
Sa_CH_Zermatt	7	123	44.0	0	0.21	17.9
Sde_CH_Audannes	8	127	42.3	1	0.20	13.5
Sde_CH_BellaLui	6	107	28.6	0	0.13	13.2
Sde_F_Galibier	8	115	36.6	0	0.17	15.4
Sde_F_Grave	7	126	50.9	0	0.24	11.5
Sde_CH_Sanetsch1	5	130	37.1	1	0.19	20.7
Sde_CH_Sanetsch2	6	111	17.1	0	0.08	26.4
Sde_F_VillardRey	7	124	44.6	0	0.22	13.2
Sdis_CH_Lukmanier	5	109	38.3	0	0.20	10.0
Sdis_CH_Nufenen	5	102	29.1	1	0.14	15.5
Sdis_CH_Schwägalp	7	107	26.9	2	0.13	21.8

In order to balance out different sample sizes, only five randomly chosen individuals per population were used to calculate diversity measurements (GenDiv and DW)

F_{tot} total present fragments, F_{Poly} percentage of polymorphic fragments, F_{Uni} unique fragments, *GenDiv* genetic diversity following Nei (1973) and *DW* "frequency-down-weighted marker values" according to Schönswetter and Tribsch (2005)

ssp. *alpina* and pH = 8.14 for *S. alpina* ssp. *depressa* (Fig. 4). The Wilcoxon Rank Test resulted in a *p* value of $1.078*10^{-09}$. The variance of the pH value was 0.07 for the six investigated *S. alpina* ssp. *depressa* populations and 0.62 for the five *S. alpina* ssp. *alpina*, suggesting that *S. alpina* ssp. *depressa* has a narrower tolerance towards soil pH than *S. alpina* ssp. *alpina*.

AFLP analyses

The three AFLP primer combinations resulted in 248 markers in total. The error rate from two replicate samples was estimated to be 8.06 % (40 out of 496 phenotypic marker comparisons were not reproducible). Markers that were non-replicable, monotypic and only present in or absent from one sample were eliminated, resulting in a final dataset of 175 polymorphic and unambiguous markers in 102 samples (DS1). On average, samples contained 87.13 AFLP bands (SD = 8.86).

Genetic diversity

Six out of fifteen populations had unique fragments (Table 2). Genetic diversity according to Nei (1973) varied from 0.08 in populations Sa_F_Seigne and Sde_CH_Sanetsch2 to 0.24 in population Sde_F_Grave (mean = 0.16, SD = 0.049). The DW value varied from 9.99 in population Sdis_CH_Lukmanier to 26.38 in population Sde_CH_Sanetsch2 (mean = 16.57, SD = 5.04).

Population structure and genetic differentiation

Twelve populations of *S. alpina* ssp. *alpina* and *S. alpina* ssp. *depressa* (DS2) were analyzed in STRUCTURE 2.3.3 (Pritchard et al. 2000). The $\log_e P(D)$ of the Bayesian clustering showed two plateaus, the first at $K = 12$ and the second at $K = 15$ (ESM 1). The variance of $K = 12$ was very high, leading to the conclusion that $K = 11$ is a more confident solution. This interpretation is supported by the calculation of Evanno's delta*K* (Evanno et al. 2005), which is highest at $K = 11$ (ESM 4). At $K = 11$ the grouping of the samples corresponded largely to the natural populations (ESM 2). At $K = 15$ the assignment of groups was widely according to their population identities except for the populations Sa_CH_Zermatt, Sa_CH_Charion and Sde_F_Grave that were split into two subgroups each (ESM 2). At $K = 2$, the grouping of populations proposed by the algorithm showed a split according to neither the taxonomic identity nor geographic proximity (ESM 3).

The AMOVA was conducted with DS2 (Table 3). Non-hierarchical AMOVA showed that the percentage of the overall variation explained 42.23 % among populations ($F_{ST} = 0.42$) and 57.77 % within populations. Hierarchical AMOVAs revealed that 4.36 % ($F_{CT} = 0.04$) of the overall variation was explained by generic subspecies separation and a non-significant variation of 1.21 % ($F_{CT} = 0.01$) by a geographic partition of the data along the Aosta break zone.

A Mantel test was conducted to test for isolation by distance. For this purpose, we first tested only the *S. alpina* ssp. *alpina* populations, then the *S. alpina* ssp. *depressa* populations and finally the *S. alpina* ssp. *alpina* and *S. alpina* ssp. *depressa* populations together. All tested combinations were not significant with *p* values between 0.55 and 0.82. This result strongly supports that no isolation by distance pattern is present.

The Principal Coordinate Analysis (PCoA) was conducted with DS2. The similarity matrix was computed using the Jaccard coefficient. Considering that the axes explain 16.18 % of the total variation and that overlaps along both axes exist, we conclude a weak differentiation of the subspecies (Fig. 5).

Discussion

Clear ecological differentiation of the subspecies

Our analyses confirmed that the morphologically distinct S. *alpina* ssp. *depressa* and *Saussurea alpina* ssp. *alpina* grow

Table 3 Analyses of molecular variance (AMOVA) based on 1,023 permutations of 85 individuals of 12 populations of *S. alpina* ssp. *alpina* and *S. alpina ssp. depressa*

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	
Among populations	11	1,016.572	10.96380	42.23	F_{ST}* = 0.42
Within populations	73	1,095.051	15.00070	57.77	
Between subspecies	1	140.471	1.15595	4.36	F_{CT}** = 0.043
Among populations within species	10	876.101	10.33837	39.02	
Within populations	73	1,095.051	15.0007	56.62	
Between W Alps and Central Alps	1	105.903	0.31496	1.21	F_{CT}*** = 0.012
Among populations within groups	10	910.669	10.79339	41.34	
Within populations	73	1095.051	15.00070	57.45	

* p value <0.001, ** p value = 0.00196 + −0.00136, *** p value = 0.15152 + −0.01257

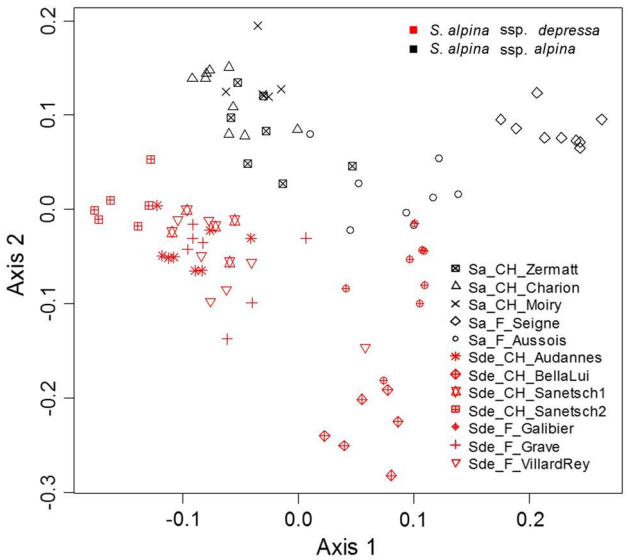

Fig. 5 Principal Coordinate Analysis (PCoA) of AFLP data. *Symbols* represent population identity. Axes explain cumulatively 16.18 % of the total variation

in divergent habitats. Ecological preferences vary in particular in relation to soil pH as well as surrounding vegetation (which is an indicator for more complex habitat parameters). According to our own observations, both subspecies, although occurring in the same mountain ranges, are never found in the same habitat. Moreover, populations of both subspecies are highly scattered in the Western Alps. As in alpine areas, a mosaic of different, mostly shallow soil types is found at a very small scale (Körner 2003), restriction and adaptation to different, island-like habitats potentially reduce gene flow among the two subspecies, but also among populations within each subspecies. Choler et al. (2004) obtained similar results in their study focusing on the local ecological and genetic differentiation of *Carex curvula* ssp. *curvula* and *C. curvula* ssp. *rosae*. The authors argue that the different ecology of the partly sympatric taxa is crucial to maintain the reproductive barrier, although they found evidence for gene flow and introgression. Given the above interpretation, we conclude from our results that *S. alpina* ssp. *alpina* and *S. alpina* ssp. *depressa* are ecologically strongly differentiated and that the preference towards different habitats, in particular soil pH, might establish a reproductive barrier, because of the isolation of suitable habitats.

High differentiation of populations overall but weak genetic differentiation of the two subspecies

Overall, *S. alpina* populations are genetically highly differentiated ($F_{ST} = 0.42$), whereas the subspecies are, in contrast to the strong ecological differentiation, only weakly differentiated ($F_{CT} = 0.04$). Rather high F_{ST} values in comparison to low F_{CT} values confirm the high degree of isolation of all investigated populations of *S. alpina*. This interpretation is also supported by the STRUCTURE analysis which showed 11 genetic clusters to be most likely. Moreover, our data evidenced neither geographical gradients of genetic diversity and DW (Table 2) nor a pattern of IBD. We argue that genetic drift in each of the populations has been the main process shaping the genetic pattern (Hutchison and Templeton 1999). Another aspect might add to the observed pattern and is related to the marker type used in our study: Gaudeul et al. (2004) has shown in a study comparing AFLP and microsatellite data, that the latter is comparably more congruent with spatial arrangements of populations. We conclude nevertheless that random genetic drift caused by unidentifiable processes like historical colonization patterns, variable gene flow among populations and other stochastic events explain the lack of any pattern within each subspecies.

We see some evidence that the two subspecies are indeed evolutionary units. The results from the PCoA as well as hierarchical AMOVA support the view of a weak, but significant differentiation. In contrast, by dividing the

populations into two geographical groups according to the Aosta break zone, a general species and allele barrier have been proposed (Fig. 1, Thiel-Egenter et al. 2011), the non-significant F_{CT} value drops to 0.01. If the different morphology and habitats of the two taxa would only be a result of a plastic response to ecological conditions (such as soil pH, slope, etc.) within a pan-population, not even a weak significant differentiation among the subspecies would be expected. Gene flow among the taxa could be an additional reason for the weak genetic differentiation among the subspecies. A similar pattern in the Alps has been detected in *Pinus mugo* ssp. *mugo* and *P. mugo* ssp. *uncinata* (Monteleone et al. 2006). Probably a combination of plasticity and weak genetic determination of subspecies is able to explain the strong ecological and morphological divergences. Hence, we show that the genetic and the ecological differentiation corresponds weakly and we hypothesize that incipient ecological plant speciation could be the major cause of the differentiation and reproductive isolation of the subspecies. Whereas *S. alpina* ssp. *depressa* is mainly found in areas in the Alps where summers are often dry and scree is abundant, *S. alpina* ssp. *alpina* is largely restricted to closed and moderately moist alpine grassland. Adaptation to dry and open habitats might be a factor resulting in the emergence of *Saussurea alpina* ssp. *depressa*. Nevertheless, an association of genetic, morphological and ecological patterns does not necessarily point out that adaptation to different habitats indeed has occurred.

We regard the rank of subspecies as appropriate as the weak genetic differentiation corresponds with ecology and morphology. The taxonomic status as subspecies is preferred over the rank as species when either the difference on the morphological level is too small or the differentiation is accompanied by occasional gene flow and/or introgression (Perny et al. 2004). Several studies that inferred horizontal gene flow between taxa and a rather weak morphological difference assigned to them the rank of subspecies (Holdálová et al. 2002; Choler et al. 2004; Slovak et al. 2012). On this basis, we favor to recognize the two taxa as subspecies (i.e., *Saussurea alpina* ssp. *alpina* and *Saussurea alpina* ssp. *depressa*), which is in line with the practice of most flora treatments in the recent past (e.g., Lauber and Wagner 2007).

Lack of a phylogeographic pattern

Weak genetic differentiation of taxa combined with the rarity of unique fragments (Table 1) has been interpreted as the result of a rather recent differentiation that may have been formed during the last glaciation cycle or even postglacially (Ehrich et al. 2008). Alvarez et al. (2009) have shown in their study that habitat ecology, especially soil acidity, affects current phylogeographical patterns in the Alps due to common migration routes during the Quaternary Ice Ages. Hence, the different habitat preferences and the distribution pattern of the subspecies might have occurred as a recent allopatric differentiation in refugia during the last glacial maximum and the subsequent dispersal along suitable habitats on either bedrock type. As our data lack any clear phylogeographical pattern we see neither support nor a rejection of this hypothesis. Since our sampling was rather limited and no samples of *S. alpina* ssp. *depressa* from the Alpes Maritimes were included, where a refugium has been suggested for *calcophilous* species (Schönswetter et al. 2005), no conclusion can be made if allopatric refugia during the last glaciation cycle did contribute in speciation.

Conclusions and outlook

Our study supports the notion that the two subspecies of *S. alpina* investigated here represent different evolutionary groups that were formed recently during the last glaciation cycles or even in postglacial periods. The ecological analyses strongly support that ecological plant speciation is a key factor in the differentiation of the taxa likely by producing and maintaining a reproductive barrier by habitat isolation. However, further studies are needed for a better understanding of the lack of a phylogeographical pattern of *S. alpina.* For the genetic analysis, samples of *S. alpina* from the Alpes Maritimes as well as a broader sampling of *S. alpina* ssp. *alpina* in the Alps with an emphasis on the Eastern Alps should be considered. Moreover, transplantation and common garden experiments might contribute to gain knowledge about the phenotypic plasticity and habitat adaptation of the taxa. A possible approach to test alternative hypotheses concerning habitat speciation processes could be contributed by the analysis of the effect of epigenetic mechanisms on the evolution of this taxon via the methylation-sensitive amplified polymorphism (MSAP) method or similar methods (e.g., Paun et al. 2010).

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